Docket No.: 4290-4000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): IHLE et al.

Group Art Unit: 1637

Serial No.: 09/870,128

Examiner: Heather Calamita

Filed: May 30, 2001

For: METHOD FOR SEPARATING NUCLEIC ACIDS INTO POPULATIONS

CLAIM TO CONVENTION PRIORITY

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In the matter of the above-identified application and under the provisions of 35 U.S.C. §119 and 37 C.F.R. §1.55, applicant(s) claim(s) the benefit of the following prior application(s):

Application(s) filed in:	United Kingdom
In the name of:	Nycomed Pharma AS
Serial No(s):	9826247.0
Filing Date(s):	November 30, 1988

☒ Pursuant to the Claim to Priority, applicant(s) submit(s) a duly certified copy of said foreign application.

☐ A duly certified copy of said foreign application is in the file of application Serial No. _____, filed _____.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

Dated: January 12, 2006

By: _____
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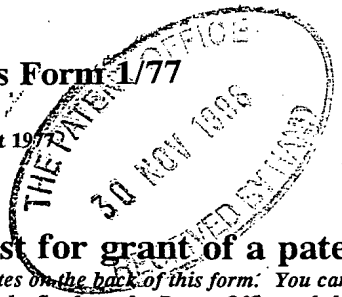

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1/77

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Request for grant of a patent

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The Patent Office
Cardiff Road
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1.	**Your reference**	28.69235
2.	**Patent application number** *(The Patent Office will fill in this part)*	30 NOV 1998 **9826247.0**
3.	**Full name, address and postcode of the or of each applicant** *(underline all surnames)*	NYCOMED PHARMA AS PO Box 4220 N-0401 Oslo Norway
	Patents ADP number *(if you know it)*	6261515003
	If the applicant is a corporate body, give country/state of incorporation	Norway
4.	**Title of the invention**	Method
5.	**Name of your agent** *(if you have one)*	Frank B. Dehn & Co.
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	179 Queen Victoria Street London EC4V 4EL
	Patents ADP number *(if you know it)*	166001

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7.	**If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application**	**Number of earlier application**	**Date of filing** *(day / month / year)*

8.	**Is a statement of inventorship and of right to grant of a patent required in support of this request?** *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	yes

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11. **I/We request the grant of a patent on the basis of this application.**

Signature **Date** 30 November 1998

12. **Name and daytime telephone number of person to contact in the United Kingdom**

Anne R. Grant
0171 206 0600

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METHOD

The present invention relates to a method of separating nucleic acid fragments. In particular, it relates to a method for separating from a population of nucleic acid molecules those which are tagged or capable of being tagged with a moiety which can be immobilised on a matrix.

Manipulation and handling of DNA is central to most biotechnology techniques. The manipulation of DNA typically involves endonuclease digestion using specific restriction enzymes which cut the DNA into fragments, followed by purification of the DNA fragments, insertion of the required fragment into cloning vectors and transfer of these vectors into non-native hosts for transcription, and optionally translation, thereby providing valuable biological information, and/or expression of the inserted DNA into a product, such as a therapeutic product. This enables, for example, eucaryotic proteins to be expressed in bacteria. The ability to cut and join DNA molecules or fragments is central to modern biotechnology. Often, these DNA fragments are generated by the polymerase chain reaction (PCR), which is now a common tool both in research and industrial biotechnology. This method enables specific DNA molecules to be amplified by means of specific short nucleic acid primers to produce large quantities of DNA which can then be further manipulated for example using the aforementioned cloning techniques.

Methods of nucleic acid manipulation which involve the use of DNA molecules generated for example by means of the aforementioned restriction enzyme cutting or by PCR are often inefficient due at least in part to the presence of unwanted DNA molecules. Such 'unwanted' molecules include for example vector DNA resulting from excision of an inserted DNA fragment from a recombinant

molecule, partially digested restriction fragments or other by-products of restriction enzyme cutting of DNA molecules, and PCR primers. The quality of primary end product DNA is crucial for the success of downstream manipulations such as ligation and transformation of bacterial or eukaryotic host cells. The ability to separate mixtures of nucleic acid molecules, such as mixtures of DNA molecules or DNA fragments into different populations and thereby remove what is considered to be the 'unwanted' or contaminating population from the desired or target nucleic acid molecule would thus enhance the efficiency of further processing or downstream steps using such generated nucleic acid molecules.

Methods for purifying nucleic acid molecules as a class are known in the art. There are however limited methods available which can separate mixtures of nucleic acid molecules such as mixtures comprising several different DNA molecules into different populations. Generally, these methods rely on separation of nucleic acid molecules, or fragments, according to size, for example by means of electrophoresis through agarose or polyacrylamide gels, followed by purification of the desired molecule or fragment. These methods have a number of drawbacks. One limiting factor is the capacity of the gel itself, which limits the amount of DNA which can be separated. The DNA needs to be visualised in the gel, generally by way of staining with ethidium bromide. Aside from being toxic to the operator, this can contribute to a reduction in the quality of the nucleic acid, so that performance in downstream applications can be poor. Recovery of nucleic acid molecules fractionated by gel electrophoresis is also inefficient leading to losses of at least 20% of DNA. Gel electrophoretic methods are also time consuming. DNA is a fragile molecule and is vulnerable to attack by exo and endonucleases. The

comparatively long process of electrophoretic separation, during which the DNA is vulnerable to degradation, can thus be detrimental to the integrity of the DNA, and affect the efficiency of downstream processes. Such separation methods are thus inefficient and costly. There is thus a need for a new method of at least partially separating nucleic acid molecules into different populations. The present invention provides such a method.

According to the present invention, there is provided a method for at least partially separating nucleic acid molecules in a sample into populations wherein a population is tagged or capable of being tagged with a moiety capable of being immobilised on a matrix, said method comprising contacting the nucleic acid containing sample with a matrix whereby the tagged molecules are captured by the matrix and thereby separated from untagged molecules.

This method is much simpler than the aforementioned electrophoretic separation method and is also quicker. It is thereby more cost effective, has greater all round efficiency, and does not suffer from the drawbacks of electrophoretic separation.

The method relies upon the tagged nucleic acid molecules being captured, i.e. immobilised or retained on the matrix, thereby effecting a separation from untagged molecules which remain in solution.

The method of the invention can be used to separate untagged nucleic acid molecules of interest (target molecules) from tagged unwanted nucleic acid molecules or fragments, in which case it is the unwanted nucleic acid molecules which are captured by the matrix, leaving the target molecules free in solution. This is advantageous where the desired target nucleic acid molecules are intended for downstream processing for example by further genetic manipulation techniques since it enables the further steps to be carried out without

the need to elute or otherwise detach the desired nucleic acid molecules from the matrix, and it also avoids the need, if necessary, to remove the tag from the nucleic acid molecules. This thus constitutes a preferred aspect of the invention. The method may however also be used to separate tagged target nucleic acid molecules of interest from untagged unwanted nucleic acid fragments, in which case it is the target molecules of interest which are captured by the matrix leaving the unwanted nucleic acid molecules in solution. Additionally, the method may be used to collect all separated nucleic acid fractions for different downstream purposes.

As used herein, 'nucleic acid molecule' refers to any nucleic acid molecule, including DNA, RNA, cDNA; and in the case of DNA, it includes double stranded and single stranded molecules, and any synthetic DNA or RNA molecule. 'Target' or 'desired' DNA refers to the nucleic acid molecule which is intended to be isolated or separated from other nucleic acid molecules. In the context of the invention, 'tag' refers to a moiety which can be attached to, bound to, incorporated in, carried by a nucleic acid molecule, or be part of the nucleotide sequence of the nucleic acid molecule, or otherwise linked to a nucleic acid molecule, and which serves as a means for capturing the tagged population of nucleic acid molecules from a nucleic acid containing sample in which one particular population is tagged in this way and other populations are not tagged. The tag thereby enables the nucleic acid mixture to be fractionated, with tagged molecules being separated from untagged molecules by means of a retention step using a matrix. The tag may be incorporated into the nucleic acid molecule, i.e. be part of the nucleic acid molecule, for example it may be part of a modified nucleotide and incorporated into the nucleic acid molecule during synthesis, or be a part of the nucleic acid sequence of

the molecule, in which case the nucleic acid molecule is itself referred to as tagged, or the tag may be attached or bound to a nucleic acid molecule, for example by post synthetic steps for example by addition of terminal nucleotides, or by binding to a recognition sequence within the nucleic acid sequence, in which case the nucleic acid, without tag attached, is described as being capable of being tagged.

The method may be used to separate or fractionate any of the aforementioned classes of nucleic acid, or mixtures of these. A preferred aspect of the invention comprises the fractionation of DNA molecules or fragments in a sample to at least partially separate the sample into different populations of nucleic acid molecules. Examples of such methods includes the separation of particular restriction fragments from a restriction enzyme digested DNA preparation, for example a PCR generated DNA molecule, or a recombinant DNA molecule.

The moiety used to tag nucleic acid molecules in accordance with the method of the invention may be any moiety which is capable of tagging a nucleic acid molecule and of immobilisation on a matrix. Immobilisation may be either by direct or indirect interaction with the matrix. The tag may thus alone be responsible for immobilisation on the matrix, or the tag may act via an intermediate or linking moiety which is responsible for interaction with the matrix, such as a binding partner for the tag.

Thus viewed from a further aspect, the present invention provides a method for at least partially separating nucleic acid molecules in a sample into populations wherein a population is tagged or capable of being tagged with a moiety capable of being immobilised on a matrix either directly or indirectly via a binding partner for the tag, said method comprising contacting the nucleic acid containing sample with a matrix, or,

where the tag interacts indirectly with the matrix by means of a binding partner, with a binding partner for the tag and with a matrix, whereby tagged molecules are captured by the matrix and thereby separated from untagged molecules.

The nature of the tag will depend at least in part upon the molecules which are to be separated and on the matrix used. Examples of suitable tags include moieties which can be incorporated into a nucleic acid molecule, for example ligands for example biotin, fluorescein or steroids or steroid like molecules such as digoxygenin, or which can be used to modify individual nucleotides within a nucleic acid molecule, and moieties such as proteins, for example proteins which have an affinity for a particular binding site within a nucleic acid molecule. The tag may thus be introduced into the nucleic acid molecule during its synthesis, for example by means of a tagged nucleotide, or after synthesis for example by addition at one end of tagged nucleotides e.g. by means of an enzymic reaction. Depending on the matrix used, the tag may interact directly with the matrix, or the tag may indirectly interact by way of a binding partner for the tag which serves to immobilise the tag on the matrix and thus acts as a linking agent. The binding partner may itself interact directly with the matrix, or it may interact by way of a further linking agent, in which case the tagged molecules may go through sequential or concurrent binding steps to enable the tagged molecule to be captured by the matrix.

In one embodiment, the tag may be a small molecule ligand. In this case, the tagged nucleic acid molecule may be immobilised on the matrix in the method of the invention by means of a binding partner to the ligand, which may be immobilised on the matrix in the form of a binding partner derivatized matrix, or which may serve as a separate linking group to immobilise the tag on the matrix.

In one embodiment of such a method, the nucleic acid sample is first contacted in solution with binding partner for the ligand, which binds only to the tagged nucleic acid molecules, and the binding partner bound tagged nucleic acid molecules are then extracted by means of a matrix with affinity for the binding partner. In another embodiment, the binding partner is first immobilised on the matrix, and is then contacted with the nucleic acid sample, retaining on the membrane only tagged nucleic acid molecules. In this embodiment, the binding partner may be immobilised onto the matrix using conventional methods appropriate for the type of matrix and the binding partner, including direct chemical bonding such as covalent bonding, adsorption, or by affinity binding.

One example of a ligand tag which may be used in the invention is biotin. Others are known in the art. Where biotin is used as the ligand, the binding partner is avidin or streptavidin, and the matrix may be one which has an affinity for proteins and is thereby able to capture streptavidin or avidin and any molecules to which (strept)avidin is bound. Avidin and streptavidin may each be used as the binding partner for biotin, and where in the following reference is made to streptavidin, the bacterial protein, it will be understood that avidin could also be used. Biotin can be readily incorporated into nucleotides, and indeed biotinylated nucleotides are available commercially. We have also found that the use of a biotin tag is very efficient in the method of the invention. Biotin accordingly represents a preferred tag for the method of the invention.

In one embodiment where biotin is used as the ligand, biotinylated nucleic acid molecules may first be incubated in solution with streptavidin, whereby streptavidin as binding partner will bind to any biotin containing nucleic acid molecules and form a binding

complex. These tagged molecules with streptavidin attached are then subsequently immobilised by means of a matrix capable of selectively immobilising proteins, whilst not being capable of immobilising nucleic acids at least under the conditions used, thereby separating out from the sample the biotin containing nucleic acid molecules.

In another related embodiment where biotin is used as the tag, the matrix itself will have streptavidin bound or attached to it. In this case, when the nucleic acid sample in solution is contacted with the matrix, biotin-tagged molecules will be retained, leaving untagged molecules free in solution.

In another embodiment, the tag may be a ligand such as fluorescin or digoxigenin or an antigen. These tags may be captured on the matrix by means of binding partners to these tags, for example an antibody to the tag, either polyclonal or monoclonal, or a fragment of such an antibody. Where the ligand is a steroid, the capturing means may be either an antibody, or a fragment thereof, or a receptor for the steroid, or a fragment thereof with steroid binding properties. These capturing means may be utilised in a similar fashion to the aforementioned use of streptavidin, with a matrix which has an affinity for proteins.

In a further embodiment of the invention, the tag may be a protein, preferably a nucleic acid binding protein, which has a specific recognition sequence within the nucleic acid molecule to be tagged. Examples of such nucleic acid binding proteins include the transcription factor AP-1 which binds to the AP-1 recognition sequence, the myb protein which binds to a specific short recognition sequence, and the lacI repressor protein, which binds to a lac operator sequence.

In a related embodiment, the tag may be viewed as a nucleic acid sequence or sequences within the nucleic

acid molecule such as a specific recognition sequence. Such sequence tags may have affinity for a protein which can be bound to a matrix. Examples include the aforementioned AP-1 recognition sequence, to which AP-1 as binding partner may bind and thereby effect binding to a protein-binding matrix. Similarly, the myb protein as binding partner may bind to a specific short recognition sequence as tag, and the lacI protein as binding partner may bind to the lac operator sequence as tag.

In such embodiments of the invention, a sample containing nucleic acid molecules which include the protein recognition sequence may be first tagged in solution by contact with the protein recognised by the specific sequence, and then the sample is contacted with the matrix whereupon tagged molecules are retained on the matrix leaving untagged molecules in solution. Alternatively, the DNA binding protein may be immobilised on the matrix, and then used to capture the nucleic acid from solution, in a similar way to the above described methods which use streptavidin.

In each of these embodiments, a protein is involved in capturing a population of nucleic acid molecules, either as the tag itself, or as the binding partner for the tag (such as antibody, or streptavidin). This is advantageous because it enables protein receptive materials to be used as the matrix, preferably materials which have selective binding for proteins and thus which do not bind nucleic acids, at least under the conditions used, thereby ensuring that untagged molecules are not captured by the membrane and sequestered from the sample. The protein may be captured by the matrix and bound to it by a variety of interactions, including ionic interaction, hydrophobic interaction and affinity binding.

The matrix may take any convenient physical form, and many are known in the art, for example sheets, gels,

filters, membranes, fibres, tubes, microtitre plates, columns, particles, and may be particulate or porous. Porous materials such as filters and membranes are convenient for separation methods according to the invention, either for filtering away unwanted tagged DNA or for collection of wanted tagged DNA. Examples include samples where an untagged population is intended for further downstream processing, and where the tagged nucleic acid population, constituting the 'undesired' population, may be captured from solution by the membrane, since it is straightforward to process the sample by filtration through the membrane offering an effective and rapid capture method and simultaneously fractionating into the filtrate the untagged nucleic acid population, thus offering a straightforward route into the next manipulation stage. Porous materials such as membranes thus constitute a preferred matrix for use in the invention.

Porous matrices may thus be conveniently used for filtering away unwanted tagged nucleic acid molecules or for collection of wanted tagged nucleic acid molecules. Such matrices may be used as part of a device for a single or multistep separation, or as part of other steps, such as for detecting, assessing or quantitating DNA or any other product in a downstream reaction stream. These porous matrices may be incorporated into separation devices such as centrifuge vials, microtitre plates, cartridges or syringes, and, depending on the sample and the downstream processes to be operated, one or more of such devices may be provided in a serial manner. Such devices may be handled manually, semiautomatically or in fully automated fashion.

In one embodiment, a NycoCard™ may be used. Where the tagged nucleic acid fragment is the sequence to be detected, it may, after binding to a protein with affinity for the tag be entrapped directly in a protein binding membrane retained in a Nycocard device. Such a

device would include an appropriate membrane with an absorbent pad such as cellulose paper placed on one side to enhance passage of the liquid sample through the membrane. Where the tagged sequences are to be eliminated, and untagged nucleic acids collected, a protein binding filter may be used as a prefilter, placed over a nucleic acid binding filter mounted in the NycoCard device so that unwanted tagged molecules are retained on the prefilter, and desired untagged molecules retained on the nucleic acid binding filter.

The matrix may be composed of a variety of materials known in the art for the purpose, including polymeric materials for example cellulose, polystyrene, agarose, latex, which may be derivatized or modified to provide means for capturing the tag itself, or for capturing the binding partner for the tag which acts as a linking agent between the tag and the matrix. Thus for example, the material may be treated eg by coating with a substance having an affinity for the tag, or the binding partner or linking agent used to mediate capture of the tag. Preferably, the matrix will have specificity for the tag or the binding partner for the tag, as compared to nucleic acid, so that untagged molecules are not retained by the matrix. In a preferred aspect of the invention, the capturing process involves a protein, either where the tag is a protein, or is a substance which has a proteinaceous binding partner which serves to link the tag to the matrix, and the matrix is thus of a protein receptive nature. Examples are known in the art and include known protein binding matrices, coated for example with polymers having a specific affinity for proteins, at least under the conditions used. The matrix may be substituted with or carry the binding partner for the tag according to methods known in the art, such as described in WO90/04786 including direct chemical bonding such as covalent bonding, adsorption, or by affinity binding.

Particularly preferred are membranes comprised of protein binding polymers, such as those described in WO98/23630,EP-0524800 and EP-0580305, for example Centriflex (TM) marketed by Edge Biosystems, USA.

The separation method of the invention is particularly convenient where a sample is to be fractionated, and untagged nucleic acid molecules in solution are to be collected for further downstream processing. The method may however also be used where it is the tagged nucleic acid molecules which are to be collected for further processing. When tagged molecules are those to be collected for downstream work, the molecules will need to be released from the matrix, and, depending on the capture method used, may also need to be released from the binding partner for the tag. The release method used may depend upon the nature of the tag, and its binding partner, and the type and strength of their mutual binding forces.

The reaction conditions chosen for the release step may also be selected so as to prevent the released tagged nucleic acid from rebinding to the matrix or to its binding partner. Examples of such methods are known in the art. Thus for example, chemical or physical conditions may be changed so as to aid release of tagged nucleic acid molecules from the matrix -bound complex. Examples of chemical methods include altering ionic strength or pH, addition of chelating agents, and the use of competing free tag molecules, or molecules chemically related thereto, molecules comprising tags or tag like moieties, molecules or ions which change the conformation of the binding partner and thereby reduce, eliminate or modify the tag-binding partner interaction, addition of detergents or dissociating agents, or by enzymatic treatment. Examples of physical methods include changes in temperature, sonication, vibration. Any combination of these physical and chemical methods may be used.

Depending on the strength of the interaction between the tag and its binding partner, it may be possible to release the tag or the tagged molecule from the binding partner or matrix by means of adding excess tag which competes with the tagged DNA for attachment sites to the binding partner for the tag or to the matrix, and the released tagged DNA may then simply be washed off. Thus where the tag is fluorescein, and the binding partner is an antibody to fluorescein, fluorescein-DNA may be released from the matrix by addition of free fluorescein in solution. Where however the tag-binding partner interaction, binding partner-matrix interaction or tag-matrix interaction is strong, addition of free tag is not always effective at disrupting the interaction. In this case, other methods such as by degrading the binding partner for example by means of pH or enzymes in such a way that it is released from the matrix and/or the tag may be used. Release may also be effected by disruption of the matrix into a form unable to bind proteins, such as by means of chemical agents, so that the protein-DNA or protein-tag-DNA complex is released, or by chemical means which disrupt the interaction or otherwise effect the affinity between protein and matrix.

The biotin-streptavidin binding pair is one where there is a particularly strong interaction and thus is not susceptible to disruption by means of addition of free biotin. Thus where the tag is biotin, and the tagged DNA is captured on the matrix by means of avidin or streptavidin, biotinylated DNA cannot be released from the membrane by addition of free biotin. This method can however be used where the tag is a biotin derivative with lower binding affinity than biotin to streptavidin, in which case the tagged DNA may be released from the matrix by addition of free biotin. This may compete with the biotinylated DNA for attachment sites to streptavidin and the released

biotinylated DNA may simply be washed off the matrix.

Depending on the tag used, it may be necessary to incorporate a step whereby the tagged DNA which is intended for downstream processing is released from the binding partner for the tag which serves to immobilise the tagged DNA on the matrix. Examples include addition of chemical substances or ions or by applying physical conditions capable of reducing the binding force between the tag and its binding partner and then collecting the released DNA.

The method of the invention can be used in a number of different applications, including analytical, preparatory and diagnostic uses, examples of which are presented below. Other applications will be apparent to those skilled in the art.

An important application of the invention is in the cutting and ligating of DNA molecules, such as in the separation of particular restriction enzyme digestion products, and the separation of ligated, circular DNA molecules from other products of ligation reactions.

Thus one application of the invention is in the manipulation of restriction enzyme digested fragments of DNA particularly where a specific fragment is required for further manipulation which thus needs to be separated from other products of the enzyme reaction. The separation method enables a population of linear DNA molecules which are tagged at one or both ends to be separated from untagged molecules. The tagged molecules may be tagged during synthesis, for example using tagged nucleotides such as biotinylated nucleotides, e.g. in the form of biotinylated primers, or the molecules may be tagged enzymically by end-labelling methods known in the art.

Thus viewed from a further aspect, the present invention provides a method for at least partially separating a mixture of restriction enzyme digested fragments of DNA wherein the starting material is a

linear DNA molecule which is tagged or capable of being tagged at one or both ends with a moiety capable of being immobilised on a matrix, said method comprising subjecting the DNA molecule to restriction enzyme digestion followed by contacting the sample with a matrix whereby the tagged molecules which originate from an end of the starting material are captured by the matrix and are thereby separated from untagged molecules.

This method is particularly well suited to separating digestion products of PCR produced DNA because of the way the synthesis works. In the PCR method of DNA amplification, two specific oligonucleotide primers are used, one of which is complementary to and therefore hybridises to the 5' end of the coding strand and one of which is complementary to and therefore hybridises to the 5' end of the noncoding strand so that in the presence of appropriate DNA polymerase enzymes, a full length copy of the target sequence can be synthesised. This copy will have the primer oligonucleotide incorporated into each of the two strands of the synthesised DNA, at the 5' end of each strand. Such PCR synthetic methods are often used to prepare specific DNA molecules or fragments for subsequent genetic manipulation, where the specific fragment of interest can be obtained by restriction enzyme cutting of a longer PCR product. As previously mentioned, these subsequent manipulations are often inefficient because of the presence in the subsequent steps such as ligation mixtures of other products from the restriction enzyme digest such as partially digested products and undigested DNA molecules. In circumstances where it is an internal fragment of the full length PCR product which is the product of interest for downstream processing, the 'unwanted' by-products of the restriction enzyme digest will include at least one terminus of the full length PCR product, the method of

the invention can be used to effect an at least partial separation of the product of interest from end-containing fragments, and undigested molecules, by utilising tagged primers for example biotinylated primers. In this way, tagged nucleic acid molecules can be removed from a nucleic acid sample, leaving in solution a sample containing the desired internal restriction fragment which, since it does not include a PCR primer, will not be tagged.

The method may also be used to separate restriction enzyme digestion products of DNA molecules which are either linear or have been linearised and which are tagged at one or both ends by means of end-labelling methods such as those which are known in the art, for example by way of enzymes. One example is the enzyme terminal deoxynucleotidyltransferase (TdT)which can be used alone or together with DNA polymerase I (Klenow fragment) to add tagged nucleotides, for example biotinylated or fluorescein or digoxygenin labelled nucleotides to the free hydroxyl groups at the 3' ends of a linear DNA molecule, thereby adding a tag to the 3' ends. In the case where the linear DNA to be subjected to restriction enzyme digestion is blunt ended or has protruding 3' ends, then only TdT is needed to tag the 3' ends. Where however the 5' end is protruding, and the 3' end is recessed, Klenow fragment is additionally needed to fill in the recessed end to enhance efficiency.

Where the tag is biotin, the sample may first be contacted with streptavidin in solution, and then the sample is passed through a protein binding membrane which binds to streptavidin and thereby retains the unwanted, tagged, nucleic acid molecules, leaving the desired fragment in solution and in a form suitable for further manipulation.

This represents a significant advance over current procedures which are laborious and involve the

separation of the restriction enzyme digestion mixture on an agarose gel, visualising the fragments by ethidium bromide staining, excising the DNA fragment of interest and purifying it from the gel which is a much more lengthy procedure and because of this involves a greater risk of exposure of the DNA to nuclease activity.

There is a further application of this aspect of the invention in the field of restriction enzyme digestion of PCR amplified products. Thus there are circumstances where it is desirable to carry out partial restriction enzyme digestion, ie with limited amounts of enzyme. Restriction enzyme preparations often contain small amounts of endonuclease which can degrade the ends of newly cut DNA, which can reduce the quality of the fragment of interest, and cause difficulties in further manipulation steps such as ligating the DNA, for example into an expression vector. Such problems caused by contaminating nucleases can however be minimised by using limited amounts of restriction enzyme and reducing the incubation periods. A drawback however is that the resulting digest will include unwanted byproducts of the restriction enzyme digestion, such as only partially cut molecules or uncut molecules. The method of the invention however enables partial digestion to be exploited, by synthesising the substrate for restriction enzyme digestion by PCR using tagged eg biotinylated primer DNA so that any DNA molecule or fragment which includes at least one terminus (such as undigested molecules, or partially digested molecules) can be removed or sequestered by means of an appropriate matrix, leaving a solution enriched in the internal restriction fragment of interest.

Another utility of the method of the invention is in excising and separating DNA fragments of interest from a recombinant molecule for further manipulation. A related aspect is in excising and separating vector DNA for use in further cloning manipulations. There is a

need for efficient methods of obtaining high quality linearised vector DNA fragments to be used in cloning or other biotechnological procedures. As is known in the art, vectors such as plasmids and viruses comprise in addition to appropriate elements for controlling replication and transcription one or more cloning sites for incorporation of heterologous DNA for propagation or expression. Just as restriction enzymes are used to insert a heterologous fragment into a cloning vector to prepare a recombinant vector, restriction enzymes are also used to excise the heterologous fragment for further genetic manipulation, or to excise the vector element for further manipulation. Such vectors comprise circular DNA molecules. These include a stuffer fragment, often a polylinker, and a fragment which includes the aforementioned control sequences. In one method, a recombinant vector having an inserted heterologous DNA fragment is digested with a restriction enzyme in order to excise the inserted DNA fragment which results in a mixture of linear DNA molecules, including the stuffer fragment of the vector itself, the insert, and also partially cut recombinant DNA molecules. The method of the invention may be used where the stuffer (or vector) element in the recombinant DNA molecule from which the heterologous insert is to be excised includes a specific protein recognition sequence, such as an AP-1 recognition sequence. In the method of the invention, the products of the restriction enzyme digestion reaction are contacted in solution with the protein for which the nucleic acid is specific, AP-1 in this case, and then passed through a protein-selective membrane which will sequester those nucleic acid molecules to which AP-1 has bound, leaving in solution only those DNA molecules which comprise the heterologous inserted DNA and thus do not have an AP-1 recognition sequence.

In another related embodiment, the method can be

used to separate the linearised form of the vector itself for further manipulation, ie a vector which has been linearised by means of restriction enzyme digestion and from which the stuffer fragment has been excised. Such a vector fragment may then be used for inserting and ligating heterologous DNA, which, following circularisation, may be used for further downstream applications such as for transformation of host cells.

In one example, the stuffer fragment is a polylinker consisting of unique restriction enzyme recognition sites. The vector is cut with one of these enzymes which will result in the molecule being linearised. The linear molecule may then be end tagged using the aforementioned enzymatic techniques. Preferably for ease of further manipulation the unique cutting enzyme is one which creates a 3' overhang. In this case, the linear vector molecule may be end tagged by the addition of tagged nucleotides such as biotinylated nucleotides by means of TdT. If the enzyme creates a 5' overhang, then Klenow fragment will additionally be needed to extend the 3' termini. We have found this method to be efficient, particularly where larger quantities of DNA are involved, greater than 10 μg. Having tagged the linearised vector, this is then subjected to further restriction enzyme digestion with one or more restriction enzymes preferably enzymes which have unique sites within the stuffer fragment one on either side of the original cutting site. In this way, end fragments are tagged and the desired fragment which is untagged may be separated by means of the method of the invention from the stuffer fragment. Thus when the sample is passed through the filter, fully cut vector will pass through the membrane.

In a further application, the method of the invention can be used to remove non-productive ligation products from a ligation mixture. The covalent joining

of two DNA fragments using the enzyme DNA ligase is central to biotechnology. In general, ligation reactions involve insertion of small DNA fragments or inserts into a larger vector DNA. It is important that the final ligation product is correctly circularised to avoid degradation in transformed host cells, such as bacterial host cells. Thus linear ligation products are of no utility and will not survive in the host bacteria. Unfortunately, ligase reactions are not efficient, resulting typically in over 80% of linear products. These lower the efficiency of the uptake of circularised, productive ligation products in E coli. From this point of view, the linear products can thus be considered to be contaminants or byproducts of the ligation reaction. The efficiency of transformation with ligated products could thus be enhanced if it were possible to remove the undesired linear ligation products from the reaction mixture prior to transformation. Currently there is no method available. Due to the polymorphic nature of the circular productive ligation products, these cannot be recovered by excision from gels. The present invention provides such a method.

Thus according to a further aspect, this invention provides a method of separating linear from circular nucleic acid molecules in a sample said method comprising introducing a tag to an end of a linear nucleic acid molecule, wherein said tag is capable of being immobilised on a matrix, by direct interaction with the matrix or by indirect interaction by means of a binding partner to the tag, and contacting the sample with a matrix or, where the tag interacts indirectly with the matrix, with the binding partner to the tag and with a matrix, whereby said tagged linear nucleic acid molecules are immobilised on the matrix.

The method of the invention is applicable in this case because all DNA molecules present in the ligation

mixture other than the desired circular molecules have free ends with exposed and reactive phosphate and hydroxyl groups. These can be end tagged by the aforementioned enzymatic methods with tagged nucleotides such as biotinylated nucleotides. Circular molecules cannot be tagged because they have no reactive 3' hydroxyl groups to which a tag could be attached. In this way, the tagged, linear molecules may be captured by the matrix and thereby separated from the ligated circularised molecules which remain in solution and can be used for further downstream processes, such as transformation of host cells.

The invention will now be described in more detail with reference to the following non-limiting Examples.

EXAMPLES

Example 1

Endlabelling of restricted DNA fragments with a 5'-protruding DNA end

Reagents:

5 µg lambda HindIII restricted DNA (Gibco 15612-13)
40 units DNA polymerase I, Large Klenow fragment (New England Biolabs #210S)
10 µl buffer for DNA polymerase I, Large Klenow fragment (New England Biolabs)
5 nmol biotin-14-dCTP (Gibco 19518-018)
5 nmol dATP (Gibco 10216-018)
5 nmol dTTP (Gibco 10219-012)
5 nmol dGTP (Gibco 10218-014)
Distilled water ad 100 µl

The reaction mixture was incubated at 25°C for 45 minutes.

The reaction mixture was passed through a S400 HR MicroSpin column (Pharmacia-Amersham 275140-01), 10 μg streptavidin (Promega #7041) was added and incubated 5 minutes at 25°C.

The reaction mixture was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to the membrane which was turned 180° and centrifuged again at 10000xg.

The supernatant was analysed on 1% agarose (FMC #50080) gel electrophoresis, stained with ethidium bromide as described in Maniatis, Molecular Cloning: a laboratory manual 2nd ed 1989.

No trace of lambda DNA could be seen on the gel indicating that the end labelled linear molecules with streptavidin attached were retained on the membrane.

Example 2

Endlabelling of a blunt-end and 3'-protruding DNA end fragments

Reagents:

2 μg Low DNA Mass™ Ladder (Gibco 10068-013)
40 units Terminal Deoxynucleotidyl Transferase, Recombinant (Gibco 10533-016)
20 μl buffer Terminal Deoxynucleotidyl Transferase, Recombinant (Gibco)
5 nmol biotin-14-dCTP (Gibco 19518-018)
Distilled water ad 100 μl

The reaction mixture was incubated at 37°C for 45

minutes.

The reaction mixture was passed through a S200 HR MicroSpin column (Pharmacia-Amersham 275120-01) and 10 μg streptavidin (Promega #7041) was added. The mixture was incubated 5 minutes at 25°C.

The reaction mixture was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to the membrane which was turned 180° and centrifuged again at 10000xg.

For inspection, the supernatant was run on 2% agarose (FMC #50080) gel electrophoresis, which was stained with ethidium bromide as described in Maniatis, Molecular Cloning: a laboratory manual 2nd ed. 1989.

No traces of DNA could be seen on the gel indicating that the end labelled linear molecules with streptavidin attached were retained on the membrane.

Example 3

Preparing vector DNA by removal of stuffer fragment

Cantab 5E vector containing a test insert (Pharmacia-Amersham 279401-01) was prepared using Qiagen maxiprep (Qiagen #12166)

Cantab 5E vector contains unique restriction sites for the enzymes SfiI, NotI and BsmI, with the SfiI and BsmI sites being located on either side of the NotI site

Reagents:
25 μg Cantab 5E vector as described above

40 units of NotI endonuclease (Boehringer-Mannheim 1014714)
5 µl buffer for NotI endonuclease (Boehringer-Mannheim 1014714)
water ad 50 µl

The mixture was incubated 1 hour at 37°C.

The reaction mixture was passed through a S400 HR MicroSpin column (Pharmacia-Amersham 275140-01) whereafter the following were added:

40 units DNA polymerase I, Large Klenow fragment (New England Biolabs #210S)
10 µl buffer for DNA polymerase I, Large Klenow fragment (New England Biolabs)
5 nmol biotin-14-dCTP (Gibco 19518-018)
5 nmol dGTP (Gibco 10218-014)
Distilled water ad 100 µl

The mixture was incubated at 25°C for 45 minutes.

The reaction mixture was passed through a S400 HR MicroSpin column (Pharmacia-Amersham 275140-01).

A sample of the reaction mixture (5 µg) was diluted to 50 µl and added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 µl distilled water was added to the membrane which was turned 180° and centrifuged again at 10000xg.

For inspection, the supernatant was run on 2% agarose (FMC #50080) gel electrophoresis, which was stained with ethidium bromide as described in Maniatis, Molecular Cloning: a laboratory manual 2nd ed. 1989.

No traces of Vector DNA were seen on the gel indicating that all the DNA was retained on the membrane.

The reaction mixture was passed through a S400 HR MicroSpin column (Pharmacia-Amersham 275140-01), and individual aliquots treated as follows:

(1)
33 µl of the reaction mixture were placed in a separate tube (1) to which was added:
20 units BsmI (Boehringer-Mannheim 1292315)
5 µl BsmI buffer (Boehringer-Mannheim 1292315)
distilled water ad 50 µl

The mixture was incubated for 1 hour at 65°C.

(2)
33 µl portion of the reaction mixture were placed in a separate tube (2) to which was added:
20 units SfiI (Boehringer-Mannheim 1288032)
5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl

The mixture was incubated for 1 hour at 50°C.

(3)
33 µl portion of the reaction mixture were placed in a separate tube (3) to which was added:
20 units SfiI (Boehringer-Mannheim 1288032)
5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl

The mixture was incubated for 1 hour at 50°C. Then the reaction mix was passed through a S400 MicroSpin column and the following were added to reaction mix:

20 units BsmI (Boehringer-Mannheim 1292315)

5 μl BsmI (Boehringer-Mannheim 1292315) buffer
distilled water ad 50 μl

The mixture was incubated for 1 hour at 65°C.

Each of the three reaction mixtures were purified on separate S400 MicroSpin columns, and 10 μg streptavidin (Promega #7041) were added to each of the mixtures which were incubated for 5 minutes at 25°C.

Each of the reaction mixtures were added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to each membrane and the membranes were turned 180° and centrifuged again at 10000xg.

The three reaction mixtures were run on 1% agarose (FMC #50080) gel, and stained with ethidium bromide.

Only from the third reaction sample could purified vector DNA be seen on the gel, samples 1 and 2 gave no detectable traces of DNA. This indicates that it is only the correct restricted vector which passed through the membrane.

Example 4

Restriction of biotinylated PCR fragment

A PCR product was produced amplifying a scFV construct using high-quality biotinylated PCR primers.

The scFV construct is an 800 base pair fragment, which has unique sites for SfiI and NotI, at 40 and 760 bases respectively.

1 μg of PCR product was mixed with 2 μg of streptavidin (Promega #7041) and incubated at 25°C for 5 minutes.

The reaction mix was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to the membrane which was turned 180° and centrifuged again at 10000xg.

For inspection, the reaction mix was run on 2% agarose (FMC #50080) gel, and stained with ethidium bromide.

No traces of the PCR product was detected, indicating that this had been retained on the membrane.

Separate aliquots were then treated as follows:

(1)
3 μg of the PCR product was added in a separate tube (1) to
20 units NotI endonuclease (Boehringer-Mannheim 1014714)
5 μl NotI buffer and
distilled water ad 50 μl
and incubated for 1 hour at 37°C.

(2)
Another 3 μg of the PCR product was added in a separate tube (2) to
20 units SfiI (Boehringer-Mannheim 1288032)
5 μl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 μl
and incubated 1 hour at 50°C.

(3)
Another 3 μg of the PCR product was added in a separate tube (3) to

20 units SfiI (Boehringer-Mannheim 1288032)
5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl
and incubated 1 hour at 50°C.

Then the reaction was passed through a S400 MicroSpin column and the following were added to the reaction mix:

20 units NotI endonuclease (Boehringer-Mannheim 1014714)
5 µl NotI endonuclease Boehringer-Mannheim 1014714) buffer
distilled water ad 50 µl
and incubated for 1 hour at 37°C.

Each of the three reaction mixtures were purified on separate S400 MicroSpin columns, and 10 µg streptavidin (Promega #7041) were subsequently added to each of the mixtures which were incubated for 5 minutes at 25°C.

Each of the reaction mixtures were added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 µl distilled water was added to each membrane, and the membranes were turned 180° and centrifuged again at 10000xg.

For inspection, the three reaction mixtures were run on 1% agarose (FMC #50080) gel, and stained with ethidium bromide.

Only from the reaction mix in tube 3 could purified PCR product be seen on the gel, tubes 1 and 2 gave no detectable traces of DNA, indicating that end-containing fragments had been retained on the membrane, and the internal fragment had passed through the membrane.

Example 5

Restriction of non-biotinylated PCR fragment

A PCR product was produced amplifying a scFV construct using high-quality biotinylated PCR primers.

1 μg of PCR product was mixed with 2 μg of streptavidin (Promega #7041) and incubated at 25°C for 5 minutes, as per Example 4.

The reaction mix was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to the membrane which was turned 180° and centrifuged again at 10000xg.

For inspection, the reaction mix was run on 2% agarose (FMC #50080) gel, and stained with ethidium bromide.

The PCR product was detected on the gel, indicating that it had not been retained on the membrane.

The following were added to 3 μg PCR product:
40 units Terminal Deoxynucleotidyl Transferase, Recombinant (Gibco 10533-016)
20 μl buffer Terminal Deoxynucleotidyl Transferase, Recombinant (Gibco)
5 nmol biotin-14-dCTP (Gibco 19518-018)
Distilled water ad 100 μl
and incubated at 37°C for 45 minutes.

The reaction mixture was passed through a S200 HR MicroSpin column (Pharmacia-Amersham 275120-01), 10 μg streptavidin (Promega #7041) was added and incubated 5 minutes at 25°C.

The reaction mixture was added toa Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugration at 10000xg for 30 seconds. 10 µl distilled water was added, the column turned 180° and centrifuged again at 10000xg.

For inspection, the reaction mixture was run on 2% agarose (FMC #50080) gel, and stained with ethidium bromide.

No PCR product could be detected on gel, indicating that it had been retained on the membrane.

Three separate aliquots were treated as follows:

(1)
3 µg of the PCR product was added in a separate tube (1) to
20 units NotI endonuclease (Boehringer-Mannheim 1014714)
5 µl NotI buffer and
distilled water ad 50 µl
and incubated for 1 hour at 37°C.

(2)
Another 3 µg of the PCR product was added in a separate tube (2) to
20 units SfiI (Boehringer-Mannheim 1288032)
5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl
and incubated 1 hour at 50°C.

(3)
Another 3 µg of the PCR product was added in a separate tube (3) to
20 units SfiI (Boehringer-Mannheim 1288032)
5 µl SfiI buffer (Boehringer-Mannheim 1288032)

distilled water ad 50 µl
and incubated 1 hour at 50°C.

Then the reaction was passed through a S400 MicroSpin column and the following were added to the reaction mix:

20 units NotI endonuclease (Boehringer-Mannheim 1014714)
5 µl NotI endonuclease (Boehringer-Mannheim 1014714) buffer
distilled water ad 50 µl
and incubated for 1 hour at 37°C.

Each of the three reaction mixtures were purified on separate S400 MicroSpin columns, and 10 µg streptavidin (Promega #7041) subsequently added and incubated for 5 minutes at 25°C.

Each of the three reaction mixtures were added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 µl distilled water was added to each membrane and the membranes were turned 180° and centrifuged again at 10000xg.

For inspection, the three reaction mixtures were run on 1% agarose (FMC #50080) gel, and stained with ethidium bromide.

Only from the reaction mix group 3 could purified PCR product be seen on the gel, tubes 1 and 2 gave no detectable traces of DNA, indicating that end containing fragments had been retained on the membrane, and the internal fragment had passed through the membrane.

Example 6

Removal of linear DNA from a population of circular and linear DNA molecules

The circular DNA starting material is the cloning vector pUC19 which has a unique HindIII site in the polylinker cloning site.

5 µg pUC19 vector DNA (New England Biolabs #301-1S) was added to:
40 units of HindIII restriction enzyme (New England Biolabs #104S)
5 µl buffer for HindIII restriction enzyme (New England Biolabs)
distilled water ad 50 µl

Incubated 1 hour at 37°C.

The reaction was passed through a S400 HR MicroSpin column and the reaction mix added to:

5 µl pUC19 vector DNA (New England Biolabs #301-1S)
40 units DNA polymerase I, Large Klenow fragment (New England Biolabs #210S)
10 µl buffer for DNA polymerase I, Large Klenow fragment (New England Biolabs)
5 nmol biotin-14-dCTP (Gibco 19518-018)
5 nmol dATP (Gibco 10216-018)
5 nmol dTTP (Gibco 10219-012)
5 nmol dGTP (Gibco 10218-014)
Distilled water ad 100 µl

The reaction mixture was incubated at 25°C for 45 minutes.

The reaction mixture was passed through a S400 HR

MicroSpin column (Pharmacia-Amersham 275140-01), 10 μg streptavidin (Promega #7041) was added and incubated 5 minutes at 25°C.

The reaction mixture was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugration at 10000xg for 30 seconds. 10 μl distilled water was added, the column turned 180° and centrifuged again at 10000xg.

For inspection, the supernatant was analysed on 1% agarose (FMC #50080) gel electrophoresis, stained with ethidium bromide as described in Maniatis, Molecular Cloning: a laboratory manual 2nd ed 1989.

Only circular vector was detected on the agarose (FMC #50080) gel, indicating that it had passed through the membrane, the linear DNA having been retained.